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ORIGINAL ARTICLE

Baseline in vivo, ex vivo and molecular responses of *Plasmodium falciparum* to artemether and lumefantrine in three endemic zones for malaria in Colombia

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Background: Colombia began using artemisinin-based combination therapies for the treatment of uncomplicated *Plasmodium falciparum* malaria in 2006. It is necessary to implement resistance surveillance to antimalarial drugs in order to promptly detect changes in parasite susceptibility. The aim of this study was to establish a susceptibility baseline of *P. falciparum* to artemether-lumefantrine using three monitoring tools.

Methods: Patients with uncomplicated malaria treated with artemether-lumefantrine underwent clinical and parasitological follow-up over 28 days. Ex vivo test was performed using the microtest technique for chloroquine, arthemeter, dihydroartemisinin and lumefantrine. *Pfmdr1* copy number and polymorphisms in *Pfk13, Pfatp6, Pfcrt* and *Pfmdr1* genes were analyzed.

Results: From a total of 150 screened patients, 49 completed follow-up for 28 days. All treated patients had adequate clinical and parasitological responses. Parasitic clearance showed a drastic reduction of parasite biomass at 24 hours and complete elimination at 48 hours. One hundred eleven isolates were processed, all exhibited high susceptibility to artemisinins and a slight decrease in susceptibility to lumefantrine. No genetic polymorphisms associated with resistance to artemisinin were found.

Conclusion: This study generated a susceptibility baseline in response to therapy with Coartem (artemether-lumefantrine) with numerical reference values, which will allow data comparison with future studies to systematically monitor changes in the parasite and to provide an early alert to the health authorities.

Keywords: Artemisinin-based combination therapies (ACT), Colombia, *Plasmodium falciparum*, Polymorphisms, Resistance surveillance, Therapeutic efficacy studies (TES)

Introduction

The current goal of the WHO Global Technical Strategy for Malaria, 2016–2030 is to promote elimination of the disease, especially in low transmission areas where resistance to antimalarial drugs has commonly originated.[1] Despite the worldwide reduction of malaria incidence and the number of fatal cases registered in recent years, in the Greater Mekong subregion where multidrug resistance has historically emerged, therapeutic efficacy failures with artemisinin-based combination therapies (ACT), such as artesunate-mefloquine, have increased.[2] This situation, so far unique to this area, has generated an epidemiological alert on the global commitment to preserve the effectiveness of artemisinin as a treatment for *Plasmodium falciparum* malaria.

In order to tackle the growing drug resistance, WHO has recommended the implementation of regular surveillance programmes employing tools such as therapeutic efficacy studies (TES),[3] ex vivo assays and molecular markers, to facilitate monitoring and containment.[4] Additionally, it has been shown that measurement of the parasitemia at 72 hours post-treatment with ACTs provides a

simple and helpful screening method to detect changes in the pattern of parasite susceptibility to artemisinins,[5] becoming currently one of the most widely used tests.

In Colombia, the Ministerio de Protección Social implemented the use of ACTs in 2006. However, in 2007 antimalarial therapies were unified with artemether-lumefantrine (ART-LUM) as the adopted combination, and it still remains so today.[6] Since the introduction of ACTs in Colombia, studies have shown high efficacy with artesunate-mefloquine[7] and artesunate-amodiaquine,[8] and low inhibitory concentrations 50 (IC_{50}) for artemisinin derivatives, such as artesunate[9] and dihydroartemisinin[10] (DHA) and partner drugs as lumefantrine (LUM),[10] suggesting appropriate responses to ACTs. However, little information is available on the therapeutic efficacy of ART-LUM combination or changes in the parasites' susceptibility or emergence of polymorphism associated with artemisinins and lumefantrine resistance.

The aim of this study was to establish the behavior baseline of *P. falciparum* populations circulating in Colombia and patients' response to treatment, using the tools available for monitoring the ART-LUM scheme in three sentinel sites. This response baseline to Coartem (artemether-lumefantrine) in Colombia will be the point of reference for subsequent measurements, allowing data comparison and giving information about necessary future changes.

Materials and methods

Geographic area and study population

Three sentinel sites were selected, taking into account the incidence of *P. falciparum* malaria in Colombia from 2009 to 2012. The sites were radically different in terms of the epidemiology and transmission of *P. falciparum*; one site was set up in the southern part of the Pacific region (Nariño), another was established in the Atlantic region (Córdoba), and the third one in southeastern Colombia (Guaviare) (Figure 1).

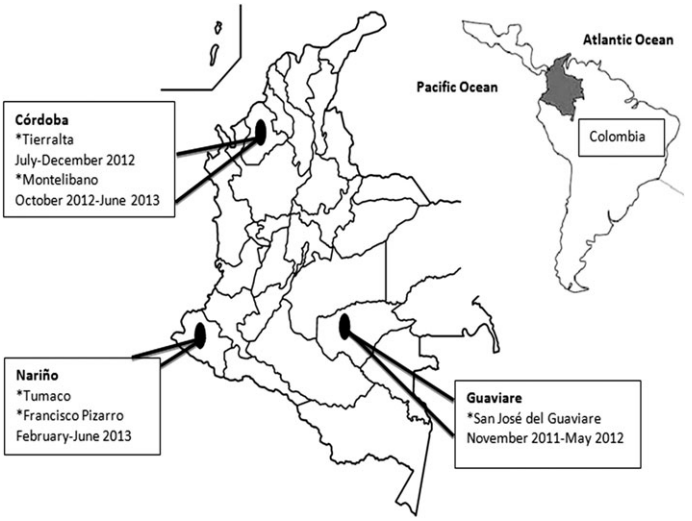

Figure 1. Sentinel sites selected to carry out the study. Three sentinel sites were chosen: one in Nariño department, which presents an annual parasitaemia index (API=10.07) and an annual *P. falciparum* index (AFI=9.58).[11] In this department the samples were collected in two municipalities: Tumaco (lat 1°49'N, long 78°52'W) and Francisco Pizarro (lat 2°0.3'37"N, long 78°39´29"W). The second sentinel site was established in Córdoba department (API=6.2; AFI=0.6)[12] and the samples were collected in the localities of Tierralta (lat 8°10'34"N, long 76°03'46"W) and Montelíbano (lat 7°59'13"N, long 75°25'30"W) and the third site, in San José (lat 2°34'15"N, long 72°38'25"W), department of Guaviare (API=6.4; AFI=0.9),[12] where like Córdoba, there is a relatively low incidence of *P. falciparum* malaria but also little information about the behavior of the *P. falciparum* parasites in response to common antimalarials. The samples were collected in San José from November 2011 to May 2012, in Córdoba from July 2012 to June 2013, and in Nariño from February to June 2013. In Tumaco, the Afro-Colombian population predominates, whereas in Tierralta and Guaviare the populations are principally Mestizo and indigenous.

Therapeutic efficacy study

Sample size was determined according to an expected proportion of 5% treatment failures in a population of infinite size, with a 95% confidence interval and +4% accuracy. A total of 150 patients were included given the likelihood of a 20% patient loss in a 28-day study. Since the epidemiological behavior of malaria is different in the three chosen departments, the sample size was proportional to the number of expected *P. falciparum* cases by locality; the estimated number of patients to be included was therefore 38, 102 and 10 for Córdoba, Nariño and Guaviare, respectively.

The selection of patients was conducted in five medical centers or diagnostic facilities belonging to the public hospital network. The selected patients met the following inclusion criteria: 1. ≥2 years of age; 2. *P. falciparum* mono-infection and uncomplicated malaria; 3. parasitemia between 250 and 50 000 trophozoites/μl; 4. absence of chloroquine in the urine (negative Saker-Solomons test):[13] 5. non-pregnant women with a negative pregnancy test (Xerion-Le Belle, IMEX group, Bogotá, Colombia), 6. patients without any concomitant chronic illness. Patients with clinical evidence of severe malaria were excluded. After the diagnosis by thick blood smears and verifying mono-infection for *P. falciparum*, 4 ml of venous blood were taken from patients who agreed to participate voluntarily in the study. Blood samples were collected in EDTA tubes and distributed as follows: 200 μl for microhematocrit, 400 μl were spotted on Whatman grade 3 MM filter paper (Brentford, United Kingdom) for molecular study, and the remaining volume was used for ex vivo study.

The patients included in the study received standard treatment (day 0) according to the Colombian guide for antimalarial treatment for uncomplicated *P. falciparum* malaria. The treatment was 4 tablets twice a day for 3 days, with the number of tablets (each containing 20 mg ART and 120 mg LUM) dependent on weight.[6] Patients were clinically supervised over 30 minutes by the medical staff after administration of the first dose to check for adverse reactions and then clinical and parasitological monitoring was conducted at days 1, 2, 3, 7, 14, 21 and 28. Endpoints considered in this study were completion of the follow-up period without treatment failure, loss to follow-up, withdrawal from study, protocol violation and treatment failure. At the end of 28 days, the patient response was evaluated according to the classification system of response to treatment as treatment success (adequate clinical and parasitological response-ACPR) and

treatment failure (early treatment failure, late clinical failure, and late parasitological failure).[6] On day 3, the presence of parasites was verified by thick blood smear and PCR.[14]

Ex vivo drug susceptibility assay

Pre-dose culture plates with antimalarial drugs

Parasites cultures in 96-well plates were tested with four antimalarial drugs: chloroquine (CQ), LUM, ART and DHA (Sigma Aldrich, St. Louis, MO, USA) at the Colombian Instituto Nacional de Salud, following the methodology described by Aponte et al.[10] For ART, a stock solution was made in ethanol. The concentration ranges of different drugs were 50–3200 nanomoles/liter (nM) for CQ, 2.5–160 nM for LUM and 0.5–32 nM for ART and DHA. Drug plate quality control was performed under the same conditions used for samples, using the reference strains T4 (Thailand)[15] and 3D7 (BEI Resources, Manassas, VA, USA),[16] which are resistant and sensitive to CQ respectively. Afterwards, the drug plates were transported under refrigeration (4–8°C) to sentinel sites, stored under the same conditions and used within two months of preparation.

Mark III schizonts maturation method (microtest)

Samples were processed at sentinel sites for a maximum of 6 hours after collection as previously described by Aponte et al.,[10] adjusting the parasitemia from 0.005 to 0.1% (250–5000 trophozoites/μl) with a 1.5% hematocrit. Two identical plates were prepared for each sample and incubated at 37°C in a standard atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The microtest was stopped when the parasites reached at least 20% schizont maturation in the positive control well (without drugs). All wells were harvested as Giemsa-stained thick smears. The percentage of schizonts was calculated as the number of schizonts (with three or more nuclei) among 200 asexual forms using light microscopy (1000X).[17] The IC_{50}, defined as the drug concentration at which parasite maturation was 50% of that measured in drug-free control wells, was calculated using a non-linear regression model with the software Hn-NonLin.[18] Microtest reading quality control was performed at the central laboratory (supplementary data).

Molecular markers

Four genes were analyzed taking into account that single nucleotide polymorphisms and increased copy numbers in the *Pfmdr1* gene have been linked to changes in the parasite susceptibility and therapeutic failure to mefloquine, artesunate, lumefantrine and quinine.[19,20] Polymorphisms in *Pfk13* (C580Y, Y493H and R539T)[21] and *Pfcrt* (K76T)[22] genes were associated with artemisinin and chloroquine resistance respectively, and the S769N *Pfatp6* mutation was associated with raised artemether IC_{50}.[23]

DNA extraction

Plasmodium DNA was extracted from blood collected on filter paper employing a commercial kit (QIAamp DNA Micro Kit, Qiagen, Hilden, Germany) following the manufacturer's instructions.

Detection of S769N allele in gene Pfatp6 *and K76T allele in gene* Pfcrt

Allele detection was carried out by PCR-RFLP. The outer and nested PCR for gene *Pfatp6* was performed following the methodology reported by Adhin et al.[24] and Ferreira et al.,[25] respectively, and for gene *Pfcrt* using the methodology described by Londono et al.[22](supplementary data). To determinate the allele in *Pfatp6*-769, the digestion was made with the enzyme AflII (New England Biolabs, Ipswich, MA, USA), while for *Pfcrt*-76 ApoI (New England Biolabs) was used. The products of PCR-RFLP were separated in agarose gels, stained with ethidium bromide (1 mg/ml) and visualized in the image analyzer UV Gel Doc XR (software Quantity One 1-D, version 4.6.2, BioRad, Hercules, CA, USA).

Sequencing of K13-propeller, Pfatp6 *and* Pfmdr1 *genes*

Pfatp6 and *Pfmdr1* genes were sequenced from field isolates with the highest and lowest IC_{50} for artemisinins (ART and DHA) and LUM, respectively. In order to determine polymorphisms in the *K13-propeller* gene, samples from day 0 (from patients who were followed until day 3) were amplified and sequenced following the methodology reported by Ménard et al.[26] The amplification of *Pfatp6* and *Pfmdr1* genes was performed according to Ferreira et al.[25] and Imwong et al.,[19] respectively. BioEdit Sequence Alignment Editor, version 7.2.5 (Ibis Biosciences, Carlsbad, CA, USA) and Codon Code Aligner, version 4.2.7, softwares were used for bioinformatic analysis. Polymorphisms associated with changes in susceptibility to antimalarials were evaluated in 14 positions of the *Pfmdr1* gene and 29 of the *Pfatp6* gene.

Estimation of Pfmdr1 *gene copy number*

Estimation of *Pfmdr1* gene copy number was performed by real-time PCR using SYBR Green DyNAmo HS kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). The primers used to amplify the *Pfmdr1* gene were described by Ferreira et al.[20] and the primers for the normalizer gene (myosin C: *PfMyoC*) were designed previously at the laboratory. The number of copies was determined by relative quantification using method $2^{-\Delta\Delta Ct}$,[20] which assumes that both target (*Pfmdr1*) and reference (*PfMyo-C*) genes are amplified with efficiencies near 100% and within 5% of each other. Once it was established that the target and reference genes have similar and nearly 100% amplification efficiencies, we determined the relative difference in expression level of the target gene in different samples. All assays were performed in duplicate in the thermocycler CFX96-BioRad detection system (BioRad, Hercules, CA, USA).

The primers' sequences and PCR programs for all molecular assays reported here, are summarized in the supplementary data.

Statistical analysis

All statistical analyses and generation of graphics were performed using Stata/SE software, version 12 (Stata Corporation, College Station, TX, USA). Ex vivo activity of each antimalarial drug was expressed as the geometric mean of inhibitory concentration 50 (GM-IC_{50}). The chi-square test was used to compare categorical variables. For all statistical tests, a p-value

equal to 0.05 was deemed significant. Fisher's exact test (two-tailed test) was used to analyze the statistical association between SNPs and drug sensitivity. The statistical association between *Pfmdr1* copy number and sensitivity to LUM was obtained by correlation coefficients.

Results

TES of artemether-lumefantrine combination

Of the 150 patients enrolled (Figure 2), only 49 (32.7%) completed the clinical and parasitological follow-up at 28 days; from these 69.4% (34/49) were men, 69.4% (34/49) were Afro-Colombians and 77.6% (38/49) were from urban areas in the municipalities studied. The median age was 25 years, ranging between seven and 75 years old, and 63.3% (31/49) of patients were older than 20 years of age. On day 0 the range of parasitemia was between 70 and 54 800 trophozoites/μl with an average of 5880 trophozoites/μl, and seven patients (14.3%) had gametocytes. Due to the reduction of malaria cases, a patient with parasitemia greater than 50 000 trophozoites/μl was included; this individual did not show any signs of complicated malaria and had strict medical supervision. The distributions of the above variables, according to the locality are shown in Table 1.

The most common symptoms were: headache 98% (48/49); chills 91.8% (45/49); sweating 85.7% (42/49); myalgias 85.7% (42/49); anorexia 83.7% (41/49); fever 77.6% (38/49). Hematocrit values ranged between 28 and 48%, with an average of 37.4% and a median of 37%.

On physical examination 12.2% (6/49) patients had hepatomegaly and 14.3% (7/49) splenomegaly (1 cm below the costal margin left/right, respectively). All patients had negative count for asexual parasites on the second day of monitoring, while sexual parasitemia persisted until day 7, and the headache, chills and fever until day 14, day 3 and day 1, respectively (Table 1). The final outcome at 28 days of follow-up in these 49 patients was classified as ACPR (i.e. clinical and parasitological resolution by the end of the follow-up period). No serious adverse events were observed after the administration of the first dose.

Evaluation of parasitemia at day 3 post-treatment

Ninety out of the 150 (60%) patients included in the study had clinical and parasitological follow-up during days 1, 2 and 3. Fifty four of them (60%) cleared the asexual parasitaemia at 24 hours and the remaining 40% (36/90) at 48 hours. PCR correction was made in 78/90 (86.6%) samples taken on day 3 with negative results, except for two samples from Tumaco, which were positive for *P. falciparum*. This result can be explained by the presence of gametocytes in the thick blood smear.

Drug susceptibility assay

A total of 111 *P. falciparum* isolates included on day 0 were processed by microtest (Figure 2). Only one (0.9%) of these samples (from Tumaco) did not achieve the required percentage of schizont maturation and another sample was not evaluated for chloroquine due to a technical failure. Geometric mean (GM) (95% CI) of the IC_{50} of the total samples analyzed for each of the drugs was 141.15 nM for CQ (95% CI 125.26 to 159.06), 7.07 nM for LUM (95% CI 6.29 to 7.96), 1.65 nM for ART (95% CI 1.44 to 1.91) and 1.18 nM for DHA (95% CI 1.03 to 1.35). Overall, the results of the three sentinel sites showed low susceptibility to CQ in 64.2% (70/109) of isolates (Figure 3). Although the studied populations have very good in vitro susceptibility to LUM, some of the analyzed isolates had a slight decrease in susceptibility. Of the 110 isolates tested, 5.4% (6/110) had IC_{50}>20 nM for LUM, one of which came from Nariño, two from Guaviare and three from Córdoba (Figure 3). In the case of ART and DHA, over 90% of isolates had IC_{50}<5 nM in both cases (ART=91.9% and DHA=95.5%) (Figure 3). $GMIC_{50}$ for CQ was greater than 100 nM in the departments of Córdoba and Nariño, being significantly higher in parasites isolated from

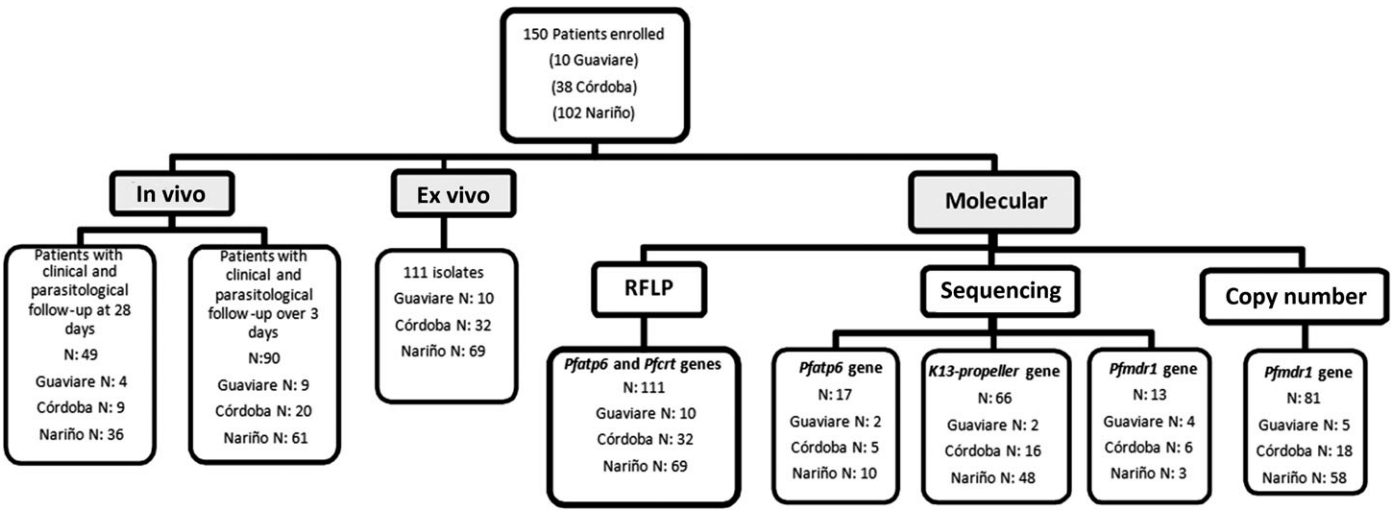

Figure 2. Flowchart describing the number of samples tested in each of the three studies. Patients enrolled and number of samples tested in the in vivo, ex vivo and molecular components of this study.

Table 1. General demographic characteristics of the population included in the study of therapeutic efficacy and parasitological follow-up

Characteristics	Study sites			
	Guaviare	Cordoba		Nariño
	San José	Montelíbano	Tierralta	Tumaco[a]
n (%) [95% CI]	4/49 (8.2) [2.3 to 19.6]	2/49 (4) [0.5 to 14]	7/49 (14) [6 to 27.2]	36/49 (73) [58.9 to 85.1]
Proportion of men (%) [95% CI]	2/4 (50) [6.8 to 93.2]	1/2 (50) [1.3 to 98.7]	6/7 (86) [42.1 to 99.6]	25/36 (69) [51.9 to 83.7]
Ethnicity n (%) [95% CI]				
Indigenous	0	0	0	1/36 (3) [0.1 to 14.5]
Mestizo	4/4 (100) [39.8 to 100]	1/2 (50) [1.3 to 98.7]	2/7 (29) [3.7 to 71]	7/36 (19) [8.2 to 36]
Afro-Colombian	0	1/2 (50) [1.3 to 98.7]	5/7 (71) [29 to 96.3]	28/36 (78) [60.8 to 90]
Asexual parasitemia average on day 0	3566	17007	4148	5856
Patients with gametocytaemia on day 0: n (%)	2/4 (50)	1/2 (50)	1/7 (14)	4/36 (11)
[b] Previous episodes of malaria n (%) [95% CI]	4/4 (100) [39.8 to 100]	2/2 (100) [15.8 to 100]	5/7 (71) [29 to 96.3]	36/36 (100) [90.2 to 100]
Negative thick film (asexual forms) n (%)				
Day 1	1/4 (25)	0/2	4/7 (57)	26/36 (25)
Day 2	3/3 (100)	2/2 (100)	3/3 (100)	10/10 (100)
Persistence of gametocytes (n)				
Day 1	1	NA	NA	NA
Day 2	1	NA	NA	NA
Day 3	NA	1	NA	3[a]
Day 7	NA	NA	1	1

[a] Two patients from Tumaco without gametocytaemia on day 0 had gametocytes on other days: one patient on day 3 and the other on day 7, without presenting sexual forms on other days of follow-up.
[b] Proportion of individuals who reported having suffered from malaria at least once prior to consultation (day 0).

Nariño (p=0.02), whereas in Guaviare *P. falciparum* populations susceptible to CQ were found (Table 2). In contrast with the results for CQ, the IC_{50} for LUM did not differ significantly among departments.

Molecular markers

Detection of S769N allele in Pfatp6 *and K76T allele in* Pfcrt

All isolates had the wild-type allele S769 in *Pfatp6*, while in *Pfcrt* all isolates were found to be mutants (76T).

Detection of SNPs in the K13-propeller, Pfatp6 *and* Pfmdr1 *genes*

For sequencing *Pfatp6, K13-propeller* and *Pfmdr1* genes, 17, 66 and 13 samples were included, respectively (Figure 2). No polymorphism was found in the *K13-propeller* gene in the region coding for the propeller domain. For *Pfmdr1* 13 isolates were sequenced, six with LUM IC_{50} above 20 nM (21.45 nM to 50.37 nM) and seven with the lowest IC_{50} (1.56 nM to 5.11 nM). Fourteen polymorphisms previously reported were analyzed, the D1246Y mutation was found in all isolates from Guaviare and Córdoba, but it was not detected in Nariño isolates, while Y184F and N1042D polymorphisms and the deletion of a repetition ATT (located in a region of low complexity of the gene) were found in all isolates analyzed. Additionally, a non-synonymous mutation G406A was identified in one isolate from Córdoba with a LUM IC_{50} of 24.3 nM (Table 3).

For *Pfatp6* the eight isolates that had IC_{50}>6 nm to ART or DHA (6.33 nM to 12.17 nM) and nine isolates that had the lowest IC_{50} for these drugs (0.5 nM to 3.48 nM) were chosen. Twenty nine polymorphisms previously reported were analyzed, only one synonymous mutation in the I898I position and the deletion of codon 847 were identified in all samples (Table 4). In addition, three polymorphisms were identified, which have not been reported previously: a deletion of six bases ranging from position 2350 to 2355 and two non-synonymous mutations at positions S466N and V307D, the latter present in 37.5% (3/8) of the samples had the highest IC_{50} for ART or DHA.

Pfmdr1 *copy number estimation*

Eighty one out of the 111 (73.0%) isolates were analyzable by qPCR: 68% of them (55/81) had a single copy of the gene, 17%

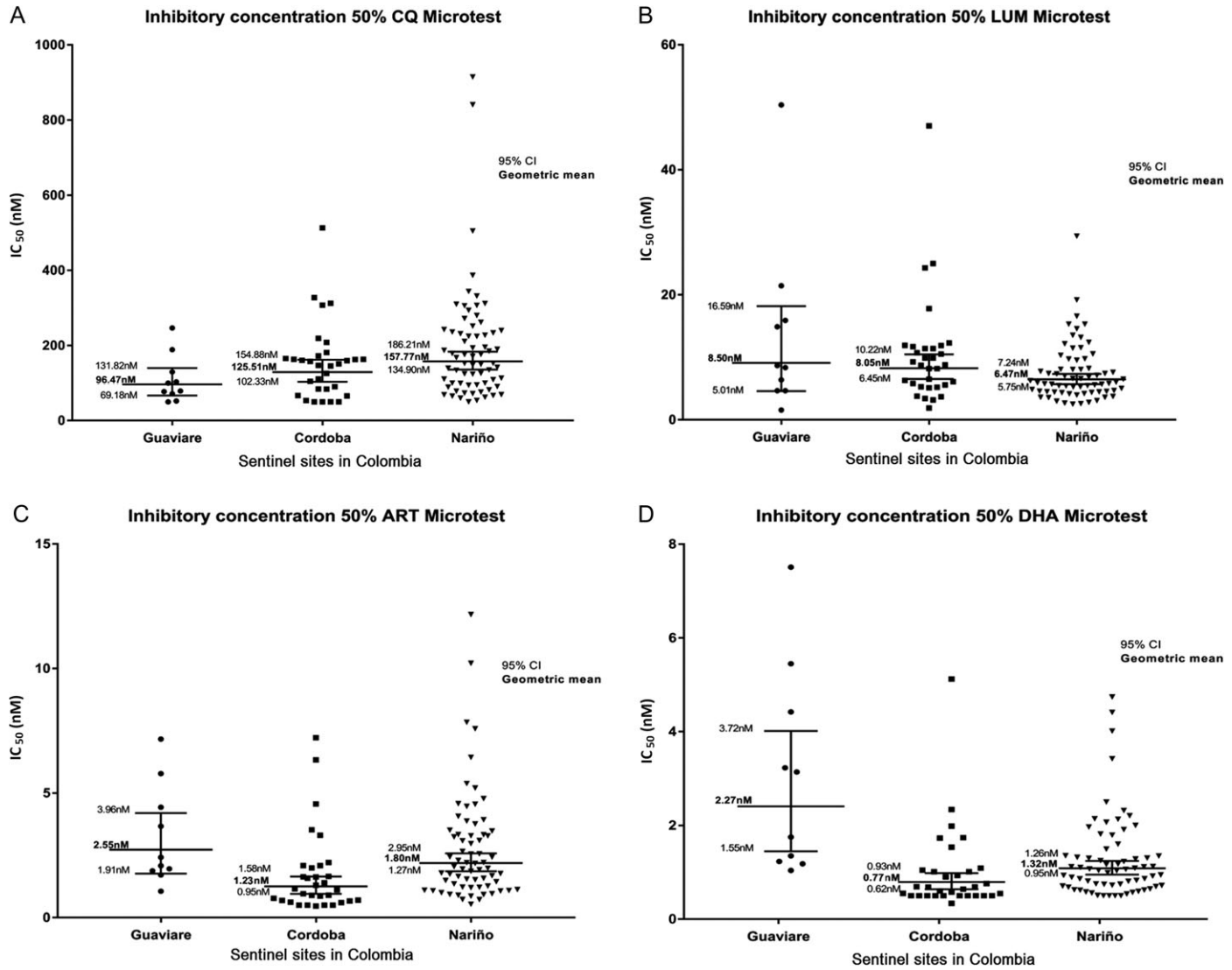

Figure 3. Ex vivo susceptibility of *P. falciparum* isolates to four antimalarial drugs, stratified by sentinel sites. The susceptibility of the parasites to A. chloroquine (CQ), B. lumefantrine (LUM), C. artemether (ART) and D. dihydroartemisinin (DHA) was determined by the schizonts maturation method Microtest.

(14/81) presented two copies, 10% (8/81) three copies and 2.5% (2/81) four and five copies (Figure 4).

Discussion

Therapeutic efficiency study results demonstrated the efficacy and safety of the ART-LUM combination in the studied populations, a situation that had been reported previously in Colombia for other ACT.[7,8] It is worth mentioning that reaching the sample size was quite difficult, due to the drastic reduction of cases of *P. falciparum* malaria that occurred between 2011 and 2012, which was 58% and 85% for Córdoba and Guaviare, respectively.[12] To improve patient recruitment, several strategies were implemented, such as including individuals with parasitemia levels below 250 trophozoites/μl, carrying out active search for patients, extending the period of inclusion, and increasing the number of locations. In addition, the high number of patients lost during follow-up contributed to not achieving an adequate sample size for TES.

The above facts demonstrate the need to implement a shorter epidemiologic strategy, such as the clinical and parasitological surveillance at day 3 post-treatment, considering that artemisinin provides a particular advantage over other treatments: rapid parasite clearance, which was demonstrated by the absence of parasites by thick blood smears in 97% of patients with parasitemia between 10 000 and 100 000 parasites/μl on day 3 post-treatment.[5] Our work showed that on day 2, no patient had asexual positive parasitemia, which allows us to recommend the implementation of clinical and parasitological follow-up at day 2 and 3 post-treatment, in order to detect early parasite clearance delays. This surveillance can be

Table 2. Ex vivo susceptibility of *P. falciparum* isolates from three sentinel sites in Colombia to four antimalarial drugs

Antimalarial Drug	Guaviare		Cordoba		Nariño	
	$GMIC_{50}$ (range)		$GMIC_{50}$ (range)		$GMIC_{50}$ (range)	
	n	nM	n	nM	n	nM
Chloroquine	10	96.47 (50–246.36)	32	125.51 (50–512.98)	68	157.77 (50–914.84)
Lumefantrine	10	8.50(1.56–50.37)	33	8.05 (1.8–47.03)	68	6.47 (2.5–29.38)
Artemether	10	2.55 (1.06–7.17)	33	1.23 (0.47–7.23)	68	1.80 (0.5–12.17)
Dihydroartemisin	10	2.27 (0.88–7.51)	33	0.77 (0.27–5.12)	68	1.32 (0.5–7.58)

IC_{50}: concentration of drug that is required for 50% inhibition in vitro; $GMIC_{50}$: geometric mean of IC_{50}; n: number of isolates tested; nM: nanomoles/liter.

Table 3. Polymorphisms found in the *Pfmdr1* gene in 13 Colombian isolates

Polymorphism	Codon	Nucleotide position	Allele: frequency n (%)	References
G406A	**G**(GGA)/**A**(GCA)	G1217C	G: 12 (92) A: 1 sample from TA (8)	Polymorphism not reported previously
N86Y	N(AAT)/Y(TAT)	A256T	N: 13 (100)	Grobusch et al.[27]
E130K	E(GAA)/K(AAA)	G388A	E: 13 (100)	Veiga et al.[28]
Y184F	**Y**(TAT)**/F**(TTT)	A553T	F: 13 (100)	Veiga et al.[28]
G249	G(GGA/GGG)	A747G	G: 13 (100)	Veiga et al.[28]
L327H	L(CTT)/H(CAT)	T980A	L: 13 (100)	Imwong et al.[19]
D587E	D(GAT)/E(GAA)	T1780A	D: 13 (100)	Imwong et al.[19]
N660 (deletion)	**N**(ATT)	1978-1980	Deletion: 13 (100)	Veiga et al.[28]
A750T	A(GCA)/T(ACA)	G2248A	A: 13 (100)	Veiga et al.[28]
S784L	S(TCA)/L(TTA)	C2350T	S: 13 (100)	Veiga et al.[28]
S1034C	S(AGT)/C(TGC)	A3106T	S: 13 (100)	Reed et al.[29]
N1042D	**N**(AAT)**/D**(GAT)	A3124G	D: 13 (100)	Reed et al.[29]
F1226Y	F(TTT)/Y(TAT)	T3677A	F: 13 (100)	Veiga et al.[28]
D1246Y	**D**(GAT)**/Y**(TAT)	G3735T	D: 3 samples from TUM (23) Y: 10 (77)	Reed et al.[29]

TA: Tierralta (Córdoba department); TUM: Tumaco (Nariño department). Polymorphisms with mutations when detected in the analyzed isolates, have been highlighted in bold.

routinely done by the national diagnostic network and would be the simplest and fastest way to detect early changes in the susceptibility of the parasite to artemisinins. These results would be the basis for proposing a TES, which will provide evidence for therapeutic failure.

On the other hand, the percentage of sexual forms in the population was nearly 20%, and Tumaco was the locality with greater persistence of gametocytes post-treatment. Although artemisinin has gametocytocidal effect on the early stages (I–III), it does not prevent the transmission of the parasite to the mosquito.[34] This may suggest the common existence of long-term infections and the need for the addition of gametocytocidal drugs such as primaquine in the ACT scheme, as a strategy to preserve the effectiveness of combination therapies and to eliminate the likelihood of reservoirs. A previous epidemiological study in Colombia highlighted the importance of using primaquine, because the elimination of gametocytes is much faster.[7]

In the ex vivo study, the evaluated isolates show high susceptibility to ART, LUM and DHA. However, it is noteworthy that for LUM, 27.2% (30/110) of the isolates had $IC_{50} > 10$ nM, of which 1.8% (2/110) were higher than 40 nM compared to the results obtained by Aponte et al. in 2006 and 2007, where the IC_{50} for LUM and ART was less than 10 nM for all isolates tested.[10] In the case of artemisinin derivatives, it was observed that more than 90% of isolates had an $IC_{50} < 5$ nM and only 1.8% (2/110) showed IC_{50} of 12.17 nM and 10.21 nM for ART. These changes in susceptibility in vitro of some isolates, which still have low IC_{50} according to the previously reported

Table 4. Analysis of polymorphisms in the *Pfatp6* gene in 17 Colombian isolates

Polymorphism	Codon	Nucleotide position	Allele: frequency n (%)	References
V307D	**V**(GTT)/**D**(GAT)	T920A	V: 13 (76) D: 4 samples, 3 with ART IC_{50}>6nM (24)	Polymorphism not reported previously
S466N	**S**(AGT)/**N**(AAT)	G1395A	S: 1 sample from SJG (6) N: 16 (94)	Polymorphism not reported previously
G786 – N787 (deletion)	**G**(CAA)**–N**(GAT)	2350-55	Deletion: 1 sample from SJG (6)	Polymorphism not reported previously
R37K	R(AGA)/K(AAA)	G110A	R: 17 (100)	Jambou et al.[30]
I89T	I(ATA)/T(ACA)	T266C	I: 17 (100)	Jambou et al.[30]
W141V	W(TGG)/V(GGG)	T421G	W: 17 (100)	Jambou et al.[30]
H243Y	H(CAT)/Y(TAC)	C727T	H: 17 (100)	Menegon et al.[31]
L263E	L(TTA)/E (GAA)	TT791–2GA	L: 17 (100)	Uhlemann et al.[32]
L402V	L(TTA)/V(GTA)	T1204G	L: 17 (100)	Menegon et al.[31]
E431K	E(GAA)/K (AAA)	G1291A	E: 17 (100)	Menegon et al.[31]
A438D	A(GCT)/D(GAT)	C1313A	A: 17 (100)	Imwong et al.[19]
Y441Y	Y(TAT/TAC)	T1323C	Y: 17 (100)	Jambou et al.[30]
D443D	D(GAT/GAC)	T1329C	D: 17 (100)	Jambou et al.[30]
N465S	N(AAT)/S(AGT)	A1394G	N: 17 (100)	Miao et al.[33]
S538R	S(AGT)/R(CGT)	A1612G	S: 17 (100)	Jambou et al.[30]
T539I	T(ACC)/I(ATC)	T1614C	T: 17 (100)	Jambou et al.[30]
S557S	S(TCT/TCA)	T1671A	S: 17 (100)	Jambou et al.[30]
N569S	N(AAT)/S(AGT)	A1706G	N: 17 (100)	Jambou et al.[30]
T570T	T(ACA/ACC)	A1710C	T: 17 (100)	Jambou et al.[30]
Q574P	Q(ACA)/P(CCA)	A1721C	Q: 17 (100)	Jambou et al.[30]
A621S	A(GCT)/S (TCT)	G1861T	A: 17 (100)	Jambou et al.[30]
A623E	A(GCA)/E (GAA)	C1868A	A: 17 (100)	Jambou et al.[23]
A630S	A(GCT)/S(TCT)	G1890T	A: 17 (100)	Menegon et al.[31]
G639D	G(GGC)/D(GAC)	G1916A	G: 17 (100)	Jambou et al.[30]
E643Q	E(GAA)/Q(CAA)	G1927C	E: 17 (100)	Jambou et al.[30]
N683K	N(AAT)/K(AAG)	T2049G	N: 17 (100)	Menegon et al.[31]
S769N	S(AGT)/N(AAT)	G2306A	S: 17 (100)	Jambou et al.[23]
E847K/deletion	**E**(GAG)/**K** (AAG)	A2536G	E : 1 sample from SJG (6), deletion: 16 (94)	Imwong et al.[19]
I885I	I(ATT/ATA)	T2656A	I: 17 (100)	Jambou et al.[30]
I898I	**I**(ATT/ATA)	T2694A	I (ATA): 17 (100)	Jambou et al.[30]
I959I	I(ATT/ATA)	T2877A	I: 17 (100)	Jambou et al.[30]
I987I	I(ATT/ATA)	T2962A	I: 17 (100)	Jambou et al.[30]

ART: artemether; SJG: San Jose del Guaviare.
Polymorphisms with mutations, when detected in the analysed isolates, have been highlighted in bold.

thresholds related to resistance,[23] reveal the importance of carefully monitoring the in vitro response of parasite populations for artemisinin and the partner drug, in order to enable timely identification of changes that compromise such therapies. Recently, it was shown that the ring-stage survival assay (RSA)[35] can assess more accurately in vitro behavior of parasites compared with conventional tests, because it measures the survival rate of parasites in stage ring to a short pulse with DHA (the active metabolite of artemisinin). One of the limitations of the present study is the lack of information regarding RSA, since this methodology was not available during the development of the work. We are aware of the need to implement this new trial in the country for routine surveillance and future resistance studies.

Although chloroquine is not part of current therapeutic regimens for *P. falciparum* malaria, it was used in Colombia until 2002. Interestingly, we observed an increase in the in vitro susceptibility to CQ of isolates circulating in Tumaco, in comparison with isolates evaluated previously.[10] Thus, the $GMIC_{50}$ to CQ using microtest in this locality was much higher previously (2006/2007=319.2/341.5 nM vs 2013=157.77 nM). This observation suggests a re-expansion of wild-type parasite populations compared with mutant parasites (K76T) in response to the absence of CQ pressure. However, all isolates with IC_{50} for CQ higher or lower than 100 nM had the 76T mutation in the *Pfcrt* gene, indicating not only fixation of alleles but the existence of other mutations in this gene or in other genes, which may be

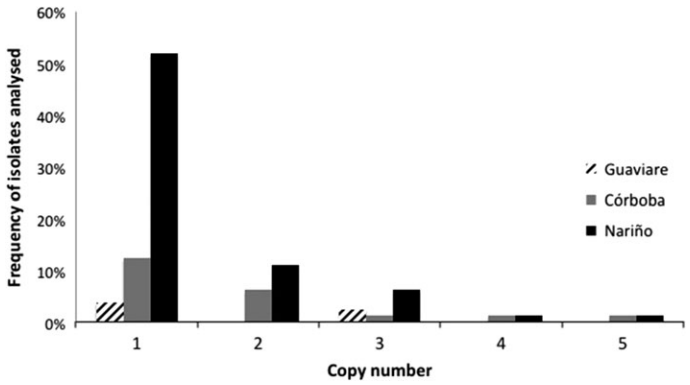

Figure 4. *Pfmdr1* copy number estimation in *Plasmodium falciparum* isolates collected from the three sentinel sites.

re-establishing susceptibility to the drug in an adaptive manner. This has previously been demonstrated in isolates from French Guiana, where the C350R mutation in *Pfcrt* is directly correlated with an increased susceptibility to CQ.[36]

Polymorphisms found in *Pfmdr1* and *Pfatp6* had no significant correlation ($p>0.05$) with the increase or decrease of the IC_{50} for the drugs evaluated. A previous study with 15 isolates from different areas of the Colombian Pacific evaluated both SNPs and copy numbers in *Pfmdr1*, finding only one copy of the gene.[37] In our study, we found that 32% (26/81) of the samples had more than one copy of *Pfmdr1*. Nevertheless, a significant correlation between copy number and IC_{50} values for the drugs tested was not found. The increase in the number of copies of *Pfmdr1* was initially described in reference strains resistant to CQ and MQ as W2[38] or clones of the Dd2 strain[39] and is correlated widely with low susceptibility to mefloquine. However, despite the fact that lumefantrine belongs to the same group of arylamino alcohols as mefloquine, no significant correlation was found, suggesting a different mechanism of action with mefloquine. Additionally, it is likely that a greater copy number of *Pfmdr1* (≥ 5) is needed to find a correlation with higher IC_{50} for DHA, as was demonstrated by Cui et al.[39] Ariey et al. found mutations in the *K13-propeller* gene strongly associated with the response to artemisinin,[21] which were evaluated in this study. None of the previously reported mutations were found, which correlates with the adequate therapeutic response and rapid parasite clearance.

In conclusion, the artemether-lumefantrine combination in the three studied localities showed a high efficacy for the treatment of uncomplicated *P. falciparum* malaria. This clinical outcome, along with parasite clearance times on the second day post-treatment and presence of parasites with wild-type genotype for the *K13-propeller* gene, constitutes the susceptibility baseline that will serve as the starting point for futures studies to systematically monitor changes in the behavior of parasites to the current first-line treatment scheme for *P. falciparum* malaria in Colombia.

Supplementary data

Supplementary data are available at Transactions Online (http://trstmh.oxfordjournals.org/).

Authors' contributions: SA and APG conceived the study; SA, CA-L, CR and MFY implemented the study in the field; CR carried out the therapeutic efficacy study; SA and CA-L performed the ex vivo testing; SDB and CG carried out the molecular study; CA-L, SDB, SA, CR, APG, CG and AK-O performed statistical analysis and interpretation of data; SA, APG, SDB and AK-O critically revised the manuscript for intellectual content. All of the authors read and approved the final manuscript. SA and APG are guarantors of the paper.

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Ethical approval: According to national and international standards, the study was approved by the Ethics Committee of the Instituto Nacional de Salud de Colombia [minutes of approval #9 on October 7, 2010]. Informed consent was obtained from the patients, who agreed to participate in the study; for underage patients, parental written consent was provided. All the results obtained were officially delivered and explained to the health authorities. The patients received anti-malarial treatment according to the national guidelines at the time of the study.

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